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Detection of neutralizing antibody against porcine epidemic diarrhea virus in subclinically infected finishing pigs

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ABSTRACT. The purpose of this study was to detect porcine epidemic diarrhea virus (PEDV) subclinically infected pigs shipped from non-case farms to slaughterhouses. Systematic sampling was conducted at two slaughterhouses. A total of 1,556 blood samples were collected from 80 case and non-case farms from pigs over 6 months old. Blood samples were centrifuged to obtain sera. Serial serum dilutions were subjected to serological examination for PEDV presence using Neutralization test (NT). The cut-off titer was set at titer of 1:2 dilution and farms with at least one positive sample in duplicate were classified as PED-positive farms. Several non-case farms (9.4%, 6/64) and 100% (16/16) of the case farms were indeed positive for PEDV. The proportion of seropositive animals from case farms was 63.7%, significantly different from that of non-case farms (4.3%, $P<0.05$). In both case and non-case farms, the proportion of seropositive animals in farrow-to-finish farms was significantly higher than in wean-to-finish farms ($P<0.05$). Seropositive animals in non-case farms were detected by NT in a sero-survey by sampling at slaughterhouses. Therefore, subclinically infected pigs should be considered prior to shipment.

KEY WORDS: passive surveillance, porcine epidemic diarrhea, subclinical infection, swine

Porcine epidemic diarrhea virus (PEDV), a member of the *Coronaviridae* family, causes acute diarrhea and dehydration in piglets, among which mortality rate can reach up to 100% [5, 16]. PEDV results in significant economic losses due to the high morbidity and mortality in neonatal piglets [1, 2, 10, 11, 17, 21]. There is an age-dependent resistance to pathogenic PEDV infection in pigs [15], where 2- and 7-day-old pigs inoculated with PEDV developed severe diarrhea and died. On the other hand, 2- and 4-week-old pigs presented with mild diarrhea and survived after inoculation with PEDV. The 8- and 12-week-old pigs did not show any clinical signs, but antibody against PEDV were detected by Neutralization test (NT) and immunofluorescence assay [15].

The virus was first identified in the United Kingdom in 1971 [20] and is now widespread throughout the United States, Canada, and many Asian countries including Japan. In Japan, PED cases were first observed in 1982 [18] and only sporadic cases were reported between 1997 and 2006, with no cases were reported from 2007 to 2012 [9]. However, large-scale PED outbreaks occurred during 2013–2015. Over 800 infected farms were identified in 38 out of 47 prefectures, resulting in at least 40,000 mortal cases, predominantly in piglets [8, 9]. Out of these cases, over 40% were confirmed in Kyushu island which possesses the largest

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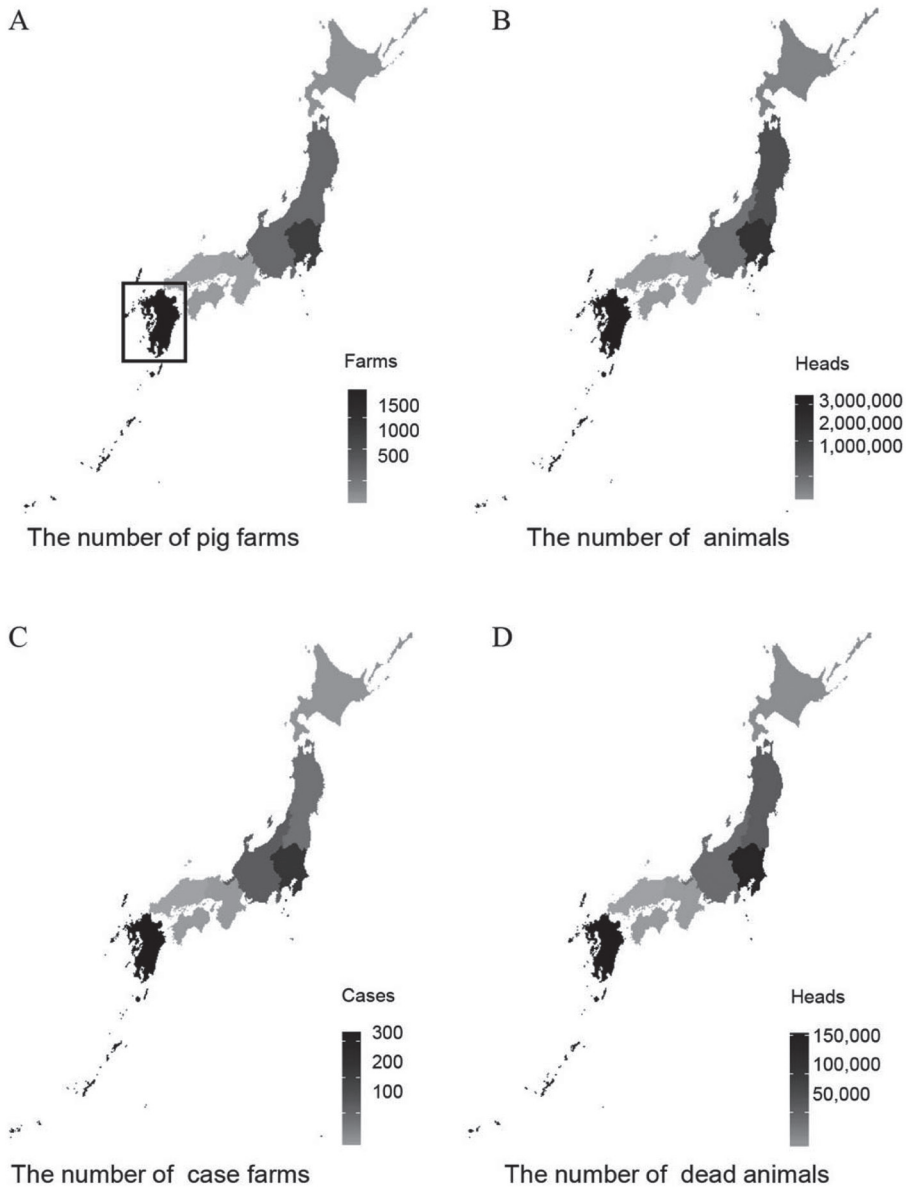

Fig. 1. Spatial distribution of pigs in Japan. (A and B) Farms and animals of pig production in 2014. (C and D) Porcine epidemic diarrhea (PED) cases and resulting number of dead animals from 2013 to 2015. The square in A indicates the Kyushu area.

number of pig farms and heads in Japan (Fig. 1). Between September 2016 and April 2017, 62 outbreaks were reported with more than 3,200 deaths [8]. To prevent the spread of PEDV between farms in Japan, some control measures were implemented. Hygienic management standards were enforced, including cleaning and disinfection of pig houses, changing clothes and boots of workers, and personnel and vehicle restriction [8]. Moreover, the use of attenuated PEDV vaccines in sows to provide maternal immunity is strongly recommended [8].

Syndromic surveillance has also been carried out as pig producers are obligated to inform farm veterinarians whenever the pigs demonstrate signs of diarrhea [8]. Subsequently, PEDV infections are confirmed by veterinary authorities at Prefectural Livestock Hygiene Centers using immunofluorescence assays and/or reverse transcriptase-polymerase chain reaction (RT-PCR) to detect PEDV antigens and the PEDV genome, respectively. Based on examination results, farms declared as PED-positive are classified under “case farms” [8]. “Non-case farms” in Japan are defined as farms with no pigs showing PED clinical signs [8]. According to the Japanese procedure for pig transportation from farms to slaughterhouses, pigs are basically shipped by trucks that are managed by logistics companies. Even though these trucks may visit different farms on the same day, the procedure requires that trucks must ship the pigs from each farm directly to the slaughterhouse before proceeding to the next farm. Loading ramps and holding pens are not separated between farms at the slaughterhouse. To prevent possible cross-contamination between case and non-case farms, different trucks are assigned to carry pigs from different farms based on their PEDV infection status. Animals from non-case farms are shipped and slaughtered in the morning, followed by case farms in the afternoon [8]. Based on this definition, if a

non-case farm has subclinical infected animals, there is a likelihood that the PEDV may spread from that premise to other non-case farms, free from PEDV, via slaughterhouse trucks. It is therefore critical to examine if farms categorized as non-case are indeed free from PEDV and that there are no subclinical infected pigs. Thus, the aim of this study was to explore the presence of PEDV subclinically infected pigs shipped from non-case farms to slaughterhouses.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from growing-finishing pigs at two slaughterhouses located on Kyushu Island. Systematic sampling was conducted for 9 days from June to July 2014. At the slaughterhouse, the samples were systematically selected from each farm at 2-pig intervals. To avoid false positive, only pigs aged over 6 months were eligible results due to maternal antibodies [12]. Moreover, the PED live vaccine had been administered in all of the farrow-to-finish farms sampled in this study [14]. Information of the infected status of all farms sampled were obtained from meat inspection office of each slaughterhouse. Based on the information, the infected status of the farms was clarified prior to sampling, where 333 samples were collected from 16 case farms and 1,223 samples were collected from 64 non-case farms. All samples were centrifuged at 2,500 × g for 5 min to obtain sera and then stored at −20°C until use.

Neutralization test (NT)

The NT method was established by the Japanese National Institute of Animal Health and is used by the Livestock Hygiene Service Center in Japan. The NT standard protocol, Vero cells (KY-5), and PEDV strain NK94P6 Tr (−) were kindly provided by the National Institute of Animal Health, Japan. Field strain, PEDV NK94P6 which belong to classical clade (G1) was obtained from a PED affected farm. Vero cells were regularly maintained in Eagle's minimal essential medium (EMEM, Sigma-Aldrich, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS, Funakoshi, Tokyo, Japan) and 0.295% (w/v) tryptose phosphate broth (TPB). PEDV was propagated in Vero cells in maintenance medium consisting of Eagle's MEM supplemented with 2% FBS and 0.295% TPB. Sera were inactivated at 56°C for 30 min prior to use. Serial two-fold dilutions of sera were mixed with an equal volume of PEDV strain NK94P6 Tr (−) suspension containing 100 × the median tissue culture infectious dose ($TCID_{50}$). The mixture was incubated for 1 hr at 37°C and then an equal volume of suspended Vero cells (approximately 30,000 cells/well) were added to each well. Following incubation for 1 week at 37°C, serum neutralization titers were calculated and expressed as the reciprocals of the highest serum dilution that inhibits cytopathic effects. The cut-off titer in this study was set at 2 in accordance with the NT method established by the Japanese National Institute of Animal Health. Farms with at least one positive sample in duplicate were classified as PED-positive farms for the purposes of this study.

Statistical analysis

The Fisher's exact test was used to assess the relationship between proportion of seropositive animals and production type. The Mann-Whitney *U*-test was used to analyze association between the mean NT titer for seropositive animals and infectious status of farms. *P* values <0.05 were considered statistically significant. All analyses were conducted using the computer programing language R (version 3.4.3; R development core team, Vienna, Austria).

Animal care and welfare

All animal protocols for this study were reviewed and approved by the Animal Ethics Committee of the University of Miyazaki's Faculty of Agriculture. During the study, animal health was monitored by licensed veterinarians and the animals were not manipulated beyond what is required for diagnostic purposes.

RESULTS

Neutralization antibody-positive animals were detected in all 16 case farms (100%) and in 6 of 64 non-case farms (9.4%; Table 1). The proportion of seropositive animals from case farms was 63.7% (212/333), significantly different from that of non-case farms (4.3% (53/1,223), $P<0.05$; Table 2). The mean NT titer for seropositive animals of case farms was not significantly different from that of non-case farms (Table 3). Within-farm level, maximum proportion of seropositive animals in case farms was 100% (18/18) while the minimum was 20.0% (6/30) (Table 2). In non-case farms, the maximum and minimum values were 90.0% (9/10) and 5.6% (1/18), respectively (Table 2). The proportions of seropositive animals in farrow-to-finish (FF) and wean-to-finish (WF) case farms were 74.0 and 57.3%, respectively, whereas they were 8.74 and 0.87% in FF and WF non-case farms, respectively. The proportion of seropositive animals in FF farms was significantly higher than in WF for both case and non-case farms ($P<0.05$).

DISCUSSION

In the present study, we determined that 9.4% of non-case farms harbored growing-finishing pigs that were positive for antibodies against PEDV. Our finding suggests that there might be PEDV infected animals in non-case farms. This means that some of the non-case farms might be not susceptible farms. Discriminating between infected and susceptible farms is critical for implementing control measures that prevent cross-contamination between farms. It was reported that trucks in which pigs had been

Table 1. Results of the farm sero-survey stratified by infectious status and production type

Infectious status	Production type	Number of farms sampled	Number of PED-positive farms	Proportion of PED-positive farms (95% CI)
Case	FF	7	7	100.0% (59.0–100.0)[a)]
	WF	9	9	100.0% (66.4–100.0)[a)]
Non-case	FF	28	5	17.9% (6.1–36.9)[b)]
	WF	36	1	2.8% (0.0–14.5)[b)]

PED, porcine epidemic diarrhea; FF, farrow-to-finish production type; WF, wean-to-finish production type. a, b) Values with different superscript letters are significantly different from case and non-case farms ($P<0.05$).

Table 2. Results of the animal sero-survey from all farms stratified by infectious status and production type

Infectious status	Production type	Number of animals sampled	Number of NT-positive animals	Minimum proportion (number of NT-positive animals/number of animals sampled)	Maximum proportion (number of NT-positive animals/number of animals sampled)	Proportion of NT-positive animals (95% CI)
Case	FF	127	94	20.0% (6/30)	100.0% (18/18)	74.0% (65.5–81.4)[a)]
	WF	206	118			57.3% (50.2–64.1)[b)]
Non-case	FF	538	47	5.6% (1/18)	90.0% (9/10)	8.74% (6.5–11.4)[c)]
	WF	685	6			0.87% (0.3–1.9)[d)]

NT, neutralization test; FF, farrow-to-finish production type; WF, wean-to-finish production type. a–d) Values with different superscript letters are significantly different from FF and WF in case and non-case farms ($P<0.05$).

Table 3. Summary statistics of the mean NT titer of seropositive animals

Infectious status	Minimum	1st quartile	Median	Mean (SD)	3rd quatile	Maximum
Case	2.00	3.65	4.59	14.99 (30.93)	9.19	128.00
Non-case	2.33	3.12	4.00	4.84 (2.64)	5.93	9.33

NT, neutralization test.

transported were contaminated with PEDV [7] and that transport vehicles are a risk factor associated with the spread of PEDV [13]. Therefore, it is important to detect subclinical infected animals in non-case farms. Our findings show a significant difference between not only infectious status (case/non-case farms), but also production type (FF/WF). On farms where there is frequent or continuous farrowing, the virus is maintained in successive generations of susceptible piglets. Thus, FF farms are more likely to have PEDV-positive animals than WF farms.

The present study established a survey to detect subclinical infections in animals from non-case farms. A passive surveillance system is currently used in Japan to detect PED status. Our results showed that some non-case farms defined as farms with no pigs demonstrating PED-like clinical symptoms indeed contain subclinical PEDV-positive animals. These results indicate that the current passive surveillance system fails to detect subclinical PEDV infection. The actual infection status in the population can be misinterpreted when subclinical infections are present. These hidden subclinical animals are ultimately overlooked. Consequently, appropriate epidemiological analyses and effective control measures are not performed. While passive surveillance can rapidly detect symptomatic disease, the sensitivity of the surveillance system is affected by many factors, such as the presence of clinical cases, attentiveness of the animal producers, and performance of the diagnostic system [4]. It is particularly difficult to detect all cases of PEDV infections by passive surveillance as some PEDV-infected animals appear healthy [3].

In Japan, PEDV infections are confirmed by histopathological diagnosis using immunofluorescence to detect the PEDV antigen and/or RT-PCR to detect the PEDV genome. However, the PEDV detection period by these methods is very short as it is limited to the acute phase only. After that, the virus is often eliminated and disappears from the infected animals [6]. In contrast, sero-surveys—such as the NT used in the present study—can detect antibodies against PEDV long after infection and can be useful in diagnosing both recent and past infections [15, 19]. The presence of infectious animals increases the risk of transmission whereas recovered animals indicates that the farm may still harbor infectious individuals capable of transmitting the virus. However, sero-surveys are unable to differentiate between infectious and recovered NT-positive cases, as both cases are positive for specific antibodies against PEDV. NT-positive animals in this study were either infectious or completely recovered with no capacity for disseminating the virus. In addition, cytopathic effect was sometimes inhibited nonspecifically by low dilution. Diagnosis of PEDV infection using NT should be considered comprehensively in conjunction with detection of PEDV or PEDV genome. RT-PCR would be more appropriate to accurately classify cases in regard to the presence of PEDV genome. Therefore, the monitoring system should utilize sero-surveys as a screening technique to enhance detection of subclinical animals. Subsequently, non-case farms with sero-positive animals should be further examined using RT-PCR to confirm if infectious animals exist. However, the cost-effectiveness of this strategy must be further evaluated. And further research will be required to confirm the sensitivity and

specificity of NT test.

This study employed a sero-survey to provide evidence regarding the presence of seropositive animals in non-case farms. Thus, the findings derived from this study are potentially important for preventive and control measures against future PED outbreaks.

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